PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS	:	Raymond A. Dwek et al.
SERIAL NO.	:	10/042,527
FILED	:	October 19, 2001
FOR	:	THERAPEUTIC COMPOSITIONS AND METHODS OF TREATING GLYCOLIPID STORAGE RELATED DISORDERS

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PETITION FOR GRANT OF PRIORITY UNDER 35 USC 119

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Dear Sir:

Applicant hereby petitions for grant of priority of the present Application on the basis of the following prior filed foreign Application:

COUNTRY	SERIAL NO.	FILING DATE
Great Britain	9909066.4	April 20, 1999

To perfect Applicant's claim to priority, a certified copy of the above listed prior filed Application is enclosed.

Acknowledgment of Applicant's perfection of claim to priority is accordingly requested.

Respectfully submitted,

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1. Your reference

NJL/P21728GB

2. Patent application number
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9909066.4

3. Full name, address and postcode of the or of each patent applicant *(underline all surnames)*

OXFORD GLYCOSCIENCES (UK) LIMITED
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Patents ADP number *(if you know it)*

7132517002

If the applicant is a corporate body, give the country/state of its incorporation

GB

4. Title of the invention

THERAPIES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent
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WC1R 4PJ

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application number	Date of filing *(day / month / year)*

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YES

a) any applicant named in part 3 is not an inventor, or

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11. I/We request the grant of a patent on the basis of this application.

Signature Date

Kilburn & Strode 20 April 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

Nicholas J Lee
Tel: 0171-539 4200

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THERAPIES

The present invention relates to compounds and agents in the manufacture of medicaments for use in the treatment of disorders which have at least a component based on glycolipid storage. Such diseases include Niemann-Pick C storage disease, Gaucher disease, Sandhoff disease, Tay-Sach's disease, GM1 gangliosidosis, Alzheimer's disease, stroke, epilepsy and cancers such as glioblastoma and astrocytoma.

The G_{M2} gangliosidoses are a group of glycosphingolipid (GSL) lysosomal storage diseases which includes Tay-Sachs disease, Sandhoff disease and G_{M2} activator deficiency (Gravel *et al* (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver *et al*) Vol 2, pp 2839-79, 3 vols, McGraw Hill, New York). They result from mutations in the genes encoding the hexosaminidase α subunit, β subunit and G_{M2} activator protein respectively. They are characterised by progressive neurodegeneration in response to high levels of lysosomal storage of G_{M2} and related GSLs, in neurones of the central nervous system (CNS) (Gravel *et al* (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver *et al*) Vol 2, pp 2839-79, 3 vols, McGraw Hill, New York). There are currently no therapies for these diseases. Potential therapeutic strategies for Tay-Sachs and Sandhoff disease include enzyme augmentation and substrate deprivation (Radin (1996) *Glycoconj. J* **13**:153-7; Platt *et al* (1998) *Biochemical Pharmacology* **56**:421-30). Augmenting the level of enzyme can be achieved using three clinical strategies, enzyme replacement, bone marrow transplantation or gene therapy.

Intravenous administration of mannose-terminated glucocerebrosidase (β-D-glycosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45) is an effective therapy for type 1 Gaucher disease, which is a non-neurological GSL storage disease (Grabowski *et al* (1995) *Ann. Intern. Med.* **122**:33-39; Beutler *et al* (1991) *Blood* **78**:1183-9). As glycoprotein enzymes fail to cross the blood-brain barrier, this is not a suitable

approach for disease involving GSL storage in the CNS. Bone marrow transplantation has the potential to increase enzyme levels in the periphery, and to a limited extent in the CNS due to secretion of enzyme from cells of bone marrow origin, including microglia (Krivit *et al* (1995) *Cell-Transplant* 4:385-392). Results of bone marrow transplantation in GSL lysosomal storage diseases involving storage in the CNS have been mixed (Hoogerbrugge *et al* (1995) *Lancet* **345**:1398-1402). Partial success was recently reported in a mouse model of Sandhoff disease given syngeneic wild type bone marrow (Norfus *et al* (1998) *J. Clin. Invest.* **101**:1881-8). This led to increased survival of the mice and improved neurological function. Gene therapy also has promise for treating these diseases, although this is currently experimental (Salvetti *et al* (1995) *Br. Med. Bull* **51**: 106-122). Substrate deprivation is a potentially generic pharmacological approach for treating the GSL storage diseases (Platt *et al* (1998) *Biochemical Pharmacology* **56**: 421-30), including the G_{M2} gangliosidoses. This strategy is based upon partial inhibition of the ceramide specific glucosyltransferase (glucosylceramide synthase, UDP-glucose:N-acylsphingosine D-glucosyltransferase, EC 2.4.1.80) which catalyses the first step in GSL biosynthesis (Sandhoff *et al* (1998) *Adv. Lipid Res.* **26**:119-142). This would reduce the levels of GSLs synthesised so they could be catabolised fully by the residual enzyme activity present in the cells.

Substrate deprivation, utilising the GSL biosynthesis inhibitor *N*-butyldeoxynojirimycin (*N*B-DNJ), has previously been tested in an *in vitro* model of Gaucher disease and shown to prevent storage (Platt *et al* (1994) *J. Biol. Chem.* **269**:8362-6). *N*B-DNJ has also been evaluated in an asymptomatic mouse model of Tay-Sachs disease and shown to reduce G_{M2} accumulation in the brain and prevent the neuropathology associated with its storage (Platt *et al* (1997) *Science* **276**:428-31). *N*B-DNJ is currently under clinical evaluation in type 1 Gaucher disease.

Defects in ganglioside biosynthesis are found in most human cancers and are thought to underlie the invasive and malignant properties of brain tumours (Hakomori 1996. *Cancer Res.* **56**:5309-5318, Fredman et al. 1996 *Glycoconj. J.* **13**:391-399).

Glycolipid metabolism also plays a critical role in other neuronal disorders, such as Alzheimer's disease and epilepsy. Niemann-Pick Type C patient neurons present with fibrillar tangles reminiscent of the morphology seen in Alzheimer's disease. Interestingly, GM1 ganglioside binding by amyloid beta-protein induces conformational changes that support its formation of fibrous polymers, and the fibrillar deposition of this protein is an early event in Alzheimer's disease (Yanagisawa et al (1995) *Nat Med* **1**:1062-6, Choo-Smith et al (1997) *Biol Chem* **272**:22987-90). Thus, decreasing GM1 synthesis could inhibit the fibre formation seen in Alzheimer's disease.

The imino sugar N-butyldeoxynojirimycin (NB-DNJ) is a potent inhibitor of alpha-glucosidase 1 (involved in N-glycan synthesis), and an even more potent inhibitor of glucosylceramide glucosyltransferase. NB-DNJ is currently undergoing clinical trials as a treatment for Gaucher and Fabry diseases, glycolipid storage disorders resulting from mutations in glucocerebrosidase and alpha-galactosidase A, respectively.

We have now found that NB-DNJ administered to mice together with glucocerebrosidase (the major therapy for Gaucher Type I patients) unexpectedly does not compromise the activity of glucocerebrosidase and provides an augmentation of enzyme activity over time due to a protective effect of NB-DNJ on the enzyme. This is surprising as the efficacy of the enzyme would be expected to be compromised in the presence of NB-DNJ as the latter is a weak inhibitor of glucocerebrosidase (IC_{50} = 0.52 mM). Furthermore, we have also found that the co-administration of NB-DNJ with bone marrow transplantation (to provide enzyme augmentation to increase the rate of neuronal glycolipid degradation) provides an unexpected synergistic effect.

Thus, in a first aspect, the present invention provides the use of an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation in the manufacture of a medicament for the treatment of a disorder which has at least a component based on glycolipid storage.

Disorders which result from accumulation/storage of glucosylceramide-containing glycolipids include Gaucher disease, Sandhoff's disease, Fabry's disease, Tay-Sach's disease, Niemann-Pick C storage disease, GM1 gangliosidosis, genetic disorders in which neuronal glycolipid accumulation contributes to the disease's pathology, e.g. mucopolysaccharidoses, neurological disorders in which glucosylceramide-containing glycolipid accumulation contributes to the disease's pathology such as Alzheimer's disease, stroke and epilepsy, cancers of neuronal origin such as glioblastoma and astrocytoma and cancers originating outside neuronal tissue but presenting with neuronal metastases.

In the context of the present invention, the term "inhibitor" is intended to include inhibitors which inhibit glucosylceramide synthesis. It includes molecules such as N-butyldeoxynojirimycin, N-butyldeoxygalactonojirimycin, N-nonyldeoxynojirimycin and other imino sugar-structured inhibitors of glucosylceramide synthesis. However, in addition, it also includes any other inhibitor of glycolipid, especially glucosylceramide, synthesis, including agents such as 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and structurally related analogues thereof. Furthermore, inhibition can also be achieved by the use of genetic approaches, based on the introduction of nucleic acid coding for proteins or peptides capable of inhibiting glycolipid synthesis or antisense sequences or catalytic RNA capable of interfering with the expression of enzymes responsible for glycolipid and especially glucosylceramide synthesis (e.g. glucosylceramide synthase). A combination of any of the above inhibitors can be used.

Agents capable of increasing the rate of glycolipid (preferably but not essentially neuronal glycolipid) degradation include enzymes which degrade glycolipids, e.g. glucocerebrosidase, lysosomal hexoseaminidases, galactosidases, sialidases and glucosylceramide glucosidase, and molecules which increase the activity of such enzymes. In addition, the agent could comprise a nucleic acid sequence (DNA or RNA) which codes for the enzymes mentioned above, i.e. such sequences could be introduced to increase natural production of such enzymes. The agent may even comprise transplanted bone marrow. A combination of the above agents can be used.

In a second aspect, the present invention provides the use of N-butyldeoxynojirimycin and an agent capable of increasing the rate of glycolipid degradation in the manufacture of a medicament for use in the treatment of a disorder which has at least a component based on glycolipid storage.

The galactose analogue of NB-DNJ, N-butyldeoxygalactonojirimycin (NB-DGJ), is known to inhibit GSL synthesis *in vitro* as effectively as NB-DNJ, but is more specific in that it does not inhibit α-glucosidase I and II or β-glucocerebrosidase (Platt et al, (1994) *J Biol Chem* **269**(43): 27108-14). It is known that only approximately 10% of the serum level of NB-DNJ is present in the cerebrospinal fluid. Accordingly, high systemic doses of NB-DNJ may have to be administered in order to achieve therapeutic levels in the CNS, and may have to be administered for the duration of a patients life. High concentrations of NB-DNJ in humans causes diarrhoea and in mice it causes weight loss and reduces the size of lymphoid organs. Thus, it would be advantageous to have an inhibitor of glucosylceramide synthesis which does not have these disadvantages of NB-DNJ.

We have now shown that, when administered to healthy mice, the distribution of NB-DGJ *in vivo* is equivalent or superior to that of NB-DNJ and inhibited GSL synthesis. In addition and significantly, NB-DGJ does not appear to cause the side effects associated with NB-DNJ.

Thus, in a third aspect, the present invention provides the use of N-butyldeoxygalactonojirimycin and an agent capable of increasing the rate of glycolipid degradation in the manufacture of a medicament for use in the treatment of a disorder which has at least a component based on glycolipid storage.

In a fourth aspect, the invention provides a product comprising an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation as a combined preparation for simultaneous, sequential or separate use in the treatment of a disorder which has at least a component based on glycolipid storage.

For example, it is envisaged that NB-DNJ (or any other inhibitor of glycolipid synthesis) can be administered to a patient with a glycolipid storage disease in order to maintain low levels of glycolipids. If the dosage of NB-DNJ is incorrect for any reason, an agent for increasing the rate of glycolipid degradation can be administered to restore the low levels of glycolipids.

In a fifth aspect, the invention provides a pharmaceutical composition comprising an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation.

Methods and processes for the production of N-butyldeoxynojirimycin can be found for example in US-A-4182767, EP-B-0012278, EP-A-0624652, US-A-4266025, US-A-4405714 and US-A-5151519 for example.

In other aspects, the present invention provides:

(a) a method for the treatment of a disorder which has at least a component based on glycolipid storage which comprises administering to a subject in need thereof a

therapeutically effective amount of an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation;

(b) a method for the treatment of a disorder which has at least a component based on glycolipid storage which comprises administering to a subject in need thereof a therapeutically effective amount of N-butyldeoxynojirimycin and an agent capable of increasing the rate of glycolipid degradation;

(c) a method for the treatment of a disorder which has at least a component based on glycolipid storage which comprises administering to a subject in need thereof a therapeutically effective amount of N- butyldeoxygalactonojirimycin and an agent capable of increasing the rate of glycolipid degradation.

The medicaments described herein and which are also for use in the methods provided herein, may include one or more of the following: preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colorants, odourants, salts, buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the compounds and/or agents described herein.

Routes of Administration

The medicaments may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such a composition may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with a carrier under sterile conditions.

Various routes of administration will now be considered in greater detail:

(i) Oral Administration

Medicaments adapted for oral administration may be provided as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions.

Tablets or hard gelatine capsules may comprise lactose, maize starch or derivatives thereof stearic acid or salts thereof.

Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

Solutions and syrups may comprise water, polyols and sugars. For the preparation of suspensions oils (e.g. vegetable oils) may be used to provide oil-in-water or water-in-oil suspensions.

(ii) Transdermal Administration

Medicaments adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis (Iontophoresis is described in *Pharmaceutical Research*, **3(6)**:318 (1986)).

(iii) Topical Administration

Medicaments adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils.

For infections of the eye or other external tissues, for example mouth and skin, a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base.

Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base.

Medicaments adapted for topical administration to the eye include eye drops. Here the active ingredient can be dissolved or suspended in a suitable carrier, e.g. in an aqueous solvent.

Medicaments adapted for topical administration in the mouth include lozenges, pastilles and mouthwashes.

(iv) Rectal Administration

Medicaments adapted for rectal administration may be provided as suppositories or enemas.

(v) Nasal Administration

Medicaments adapted for nasal administration which use solid carriers include a coarse powder (e.g. having a particle size in the range of 20 to 500 microns). This can be administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nose from a container of powder held close to the nose.

Compositions adopted for nasal administration which use liquid carriers include nasal sprays or nasal drops. These may comprise aqueous or oil solutions of the active ingredient.

Medicaments adapted for administration by inhalation include fine particle dusts or mists, which may be generated by means of various types of apparatus, e.g. pressurised aerosols, nebulisers or insufflators. Such apparatus can be constructed so as to provide predetermined dosages of the active ingredient.

(vi) Vaginal Administration

Medicaments adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

(vii) Parenteral Administration

Medicaments adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions. These may contain antioxidants, buffers, bacteriostats and solutes which render the compositions substantially isotonic with the blood of an intended recipient. Other components which may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of a sterile liquid carrier, e.g. sterile water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

Dosages

Dosages will be readily determinable by routine trials, and will be under the control of the physician or clinician. The guiding principle for determining a suitable dose will be delivery of a suitably efficacious but non-toxic, or acceptably toxic, amount of material. For NB-DNJ or a similar compound, a daily dosage for an adult could be expected to be in the range of from 1 mg to 2 g of active agent, and may be in the range of from 100 to 800 mg or 300 to 600 mg. The dosage may be administered in a single daily dose or alternatively in two, three or more doses during the day.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*.

In the accompanying drawings:

Figure 1 is a graph plotting % survival against age of Sandhoff mice in days when treated with different agents.

Figures 2A-D are graphs showing the short term distribution of radiolabelled *N*B-DNJ and *N*B-DGJ in mouse. Mice (n = 5 per group) were dissected 90 min after oral administration of [^{14}C]-*N*B-DNJ (open bars) or [^{3}H]-*N*B-DGJ (filled bars). A = total compound in intestine and urine. B = total compound in organs. C = compound concentration in serum. D = compound in organs expressed as a ratio to compound in serum. * denotes a significant difference between the *N*B-DNJ and the *N*B-DGJ treated mice ($p<0.05$).

Figures 3A-C show glycosphingolipid depletion in mouse liver after feeding *N*B-DNJ or *N*B-DGJ. Gangliosides were purified from liver and separated by TLC. G_{M2} concentration was measured by densitometry of the scanned TLC chromatograms. A = G_{M2} concentration in livers of mice fed 300 – 4800 mg/kg/day *N*B-DNJ (open bars) or *N*B-DGJ (filled bars) for 10 days, (n = 5 per group). B = TLC separated G_{M2} band of livers from mice treated for 5 weeks with 2400 mg/kg/day. C = densitometry of TLC in B. * denotes significantly lower concentration than the control concentration ($p<0.05$).

Figure 4 shows the growth of mice fed *N*B-DNJ or *N*B-DGJ. Mice were given 2400 mg/kg/day of *N*B-DNJ (o), *N*B-DGJ (•), or a control diet (▫). N = 10 per group. * denotes a significant difference compared to control weights ($p<0.01$).

Figure 5 shows the lymphoid organ size in mouse after *N*B-DNJ or *N*B-DGJ treatment. Wet weight of thymus and spleen was determined at dissection after 5 weeks of treatment with 2400 mg/kg/day of *N*B-DNJ (open bars). *N*B-DGJ (filled bars), or a control diet (dashed bars). N = 4 per group. * denotes a significant difference compared to control weights ($p<0.001$).

Figure 6 shows the inhibition of lactase activity by *N*B-DNJ, *N*B-DGJ, DNJ, and DGJ. Lactase activity expressed as % of control activity at different concentrations of *N*B-DNJ (o), *N*B-DDJ (•), DNJ (▫), and DGJ (■).

The invention will now be described with reference to the following examples, which should not in any way be construed as limiting the scope of the invention.

EXAMPLES

Example 1 – Co-administration of Ceredase™ and NB-DNJ

A group of mice were treated with NB-DNJ at 4800 mg/kg/day for 5 weeks. After a low intravenous dose (5-10 U/kg) of Ceredase™ (Genzyme Corporation) administered as a single injection via the tail vein, serum enzyme activity was measured by taking sequential serum samples from the tail vein to monitor enzyme activity over time. Ceredase™ is a modified form of β glucocerebrosidase. The results are shown in Table 1 below.

Table 1 – Effect of NB-DNJ on circulatory activity and half life of Ceredase™

Mouse		Peak Activity	$T_{1/2}$ (min)
Control	1	5.8	4.2
	2	7.9	3.3
	3	8.0	1.5
	4	6.8	1.8
	5	30.0	1.4
	6	2.8	2.0
	7	13.6	1.2
	8	17.6	1.2
Mean ± sem		11.6 ± 3.1	2.1 ± 0.4
NB-DNJ	1	13.9	1.7
	2	32.1	4.9
	3	24.1	5.3
	4	13.1	3.0
	5	21.0	3.5
	6	68.3	2.4
	7	19.2	2.8
Mean ± sem		27.4 ±7.2	3.4 ± 0.5

Ceredase activity and serum half lives appeared to be increased in mice treated with NB-DNJ, suggesting a protective effect of the compound to enzyme clearance. Therefore, (a) co-administration of NB-DNJ with Ceredase™ does not compromise activity and (b) there is a surprising augmentation of enzyme activity over time due to a protective effect of the compound on the enzyme.

Example 2 – Co-administration of NB-DNJ and Bone marrow transplantation in a mouse model of Sandhoff disease

Sandhoff mice were bone marrow transplanted at two weeks of age and drug therapy initiated at 9.5-11 weeks of age (600 mg/kg/day). Survival curves were plotted for each group of animals with each point on the graph representing a death (see Figure 1). The untreated (no BMT, no drug) survived (longest survivor) until 140 days (filled circles), NB-DNJ only (no BMT) survived until 170 days, BMT only (no NB-DNJ) survived until 200 days, and NB-DNJ plus BMT had extended survival from 200-280 days. The data show synergy approximately 13% above additive.

In Examples 3-7 below, the following materials and methods were used:

Animals

Female C57BL/6 mice were housed under standard non-sterile conditions. The mice were provided with water *ad libitum* and prior to drug administration were fed pelleted chow (expended Rat and Mouse Chow 1, SDS Ltd., Witham, Essex, UK). All experiments were performed on age-matched animals.

Treatment of Mice with NB-DNJ and NB-DGJ

The mice (6 weeks old) were fed a diet of powdered chow (expended Rat and Mouse Chow 3, ground, SDS Ltd.) or diet containing *N*B-DNJ or *N*B-DGJ. The diet and compound (both as dry solids) were mixed thoroughly, stored at room temperature, and used within 7 days of mixing. The mice were maintained on *N*B-DNJ or *N*B-DGJ at doses of 300 – 4800 mg/kg/day for 10 days, or 2400 mg/kg/day for 5 weeks.

Radiolabelling of NB-DGJ

A galactose oxidase/Na[^{3}H]$_4$B method was used to radiolabel the C6-carbon of *N*B-DGJ. A solution of *N*B-DGJ (1.3 mg), galactose oxidase (80 units), and catalase (37000 units) in 200 µl 10 mM sodium phosphate buffer was incubated for 24h at room temperature whilst

stirring. The reaction was stopped by heating the solution to 95°C for 5 min. After centrifuging (10 mins, 13000 rpm), 1M NaOH was added to the supernatant until pH 10-12 was achieved. Na[^{3}H]$_4$B (4.3 mCi) was added and the solution incubated for 2h at 30°C, after which $NaBD_4$ (1 mg) was added and the solution incubated for 1h at 30°C. The solution was neutralised with 1M acetic acid and then dried down. After removing borate by washing with acidified methanol (0.6% glacial acetic acid in methanol) 5-10 times, the [^{3}H]-*N*B-DGJ mixture was resuspended in water, added to an AG50-column (equilibrated with water) and eluted with 1-4 M NH_3. [^{3}H]-*N*B-DGJ was further purified on HPLC (Dionex CS10 hpcec chromatography, isocratic elution with 50 mM Na_2SO_4, 2.5 mM H_2SO_4, 2.5 mM H_2SO_4, and 5% ACN), and finally the AG50-column step was repeated.

Short-term Distribution of [^{14}C]-NB-DNJ and [^{3}H]-NB-DGJ in Mice

Mice were orally gavaged with 100 µl water containing 25µg (10^6 cpm) [^{14}C]-*N*B-DNJ or [^{3}H]-*N*B-DGJ and 1 mg non-radiolabelled *N*B-DNJ or *N*B-DGJ, respectively. Urine and faeces were collected over 90 min. After 90 min the mice were killed and the serum, organs, and any additional urine and faeces were collected. Organs were homogenized in a four fold volume of water and faeces in a ten fold volume. Aliquots of 500 µl homogenate, 100 µl urine, or 50 µl serum were mixed with 4 ml scintillation fluid and [^{14}C] or [^{3}H] counts measured. The quenching by the different tissues of both isotopes was determined by measuring the counts of known amounts of radiolabelled compound added to tissue homogenates, and the results were corrected accordingly.

Glycosphingolipid Analysis of Mouse Liver

Liver samples were homogenised in water and lyophilised. Dried homogenates were extracted twice in chloroform: methanol (2:1, v/v), first overnight at 4°C and then for 3h at room temperature, pooled and dried under nitrogen. The extracts were resuspended in 500 µl chloroform: methanol (1:1, v/v), base-treated by adding 83 µl of 0.35 M NaOH in methanol and digested for 90 min at room temperature and partitioned by adding 83 µl water: methanol (9:1, v/v), 166.5 µl water and 416 µl chloroform. The upper phase

containing the gangliosides was separated from the lower phase after mixing and low speed centrifugation, and the lower phase was washed twice with Folsh (chloroform: methanol: 0.47% KCl, 3:48:47, v/v). Upper phases were combined, dried down to half volume under nitrogen, dialysed against water, lyophilised and resuspended in chloroform: methanol (2:1, v/v). An equivalent of 5 mg dry weight of tissue was separated by TLC chloroform: methanol: 0.22% $CaCl_2$, 60:35:8, v/v). The TLC plate was air-dried, sprayed with orcinol: sulphuric acid (0.2% (w/v): 2N), and heat-treated (90°C for 10 min). The intensity of bands was quantified by scanning densitometry.

Determination of NB-DNJ and NB-DGJ Concentrations in Serum and Liver

Serum and supernatant of liver homogenate (130 mg/ml in 10% methanol) were centrifuged three times through a Millipore Ultrafree filter, after an internal standard (*N*B-pentylDNJ) had been added to the samples. The pooled filtrates were purified on an HCl preconditioned SCX column, eluted with 1% NH_3 in MeOH, dried down, resuspended in water, further purified on a C18 column (MeOH preconditioning, H_2O wash, and MeOH elution), and finally quantified by HPLC (Dionex CS10 hpcec chromatography, isocratic elution with 50 mM Na_3SO_4, 2.5 mM H_3SO_4, and 5% ACN).

Purification of Disaccharidases and Measurement of Sucrase, Maltase and Lactase Activity

The enzymes sucrase-isomaltase (EC 3.2.1.10/48) and lactase-phlorizin hydrolase (EC 3.2.1.62/108) were purified from porcine intestine at 4°C as follows. The intestine (100g) was cut into small pieces, washed by stirring in 250 ml of 150 mM NaCl/10 mM KCl for 30 min, and extracted twice with 125 ml of 2M urea, 50 mM EDTA, and 50 mM KCl at pH 7. The urea extracts were combined and homogenised (Waring blender), the homogenate was centrifuged at 60,000g for 75 min, and the pellet was resuspended in 50 ml of a solution containing 10 mM EDTA and 10mM L-cysteine-HCl in 50 mM potassium phosphate buffer at pH 7.5 (pre-equilibrated to 37°C). After addition of papain (15 units/ml), the mixture was incubated for 30 min at 37°C, and centrifuged at 105000g

for 60 min. The supernatant was removed and precipitated in 75 ml of ethanol at -20°C for 1h. The precipitate was recovered by centrifugation at 5000g for 10 min, dissolved in 5-10 ml of 10 mM potassium phosphate buffer at pH 7.5, and the solution was centrifuged at 30000g for 60 min. The supernatant was removed and stored at 4°C in the presence of 0.02% sodium azide. Sucrase, maltase and lactase activity were determined in the enzyme preparation (diluted to a suitable concentration) by incubating 50 µl enzyme. 125 µl sodium citrate buffer (60 mM, pH 6), and 125 µl disaccharide substrate at 37°C for 30 min, heating to 100°C for 3 min to inactivate the enzyme centrifuging the mixture at 13000g for 10 min, and determining the glucose concentration by adding 50 µl of the supernatant to 1 ml trinder reagent (Sigma) and reading the absorbance at 505 nm after 18 min.

Statistical Analysis

Conventional statistical methods were employed to calculate mean values and standard errors of the mean (S.E.M.). Differences between groups of mice were tested for significance using Student's t-test for unpaired observations. Results in the text and tables are presented as means ± S.E.M.

Example 3 – Short-term Distribution of [^{3}H]-NB-DGJ and [^{14}C]-NB-DNJ in Mice

The short-term distribution of *N*B-DGJ and *N*B-DNJ in mice was determined by giving the compounds to mice by oral gavage. The radioactive counts in organs, serum, faeces and urine were measured after 90 min. The concentration of *N*B-DNJ was 28% higher than that of *N*B-DGJ in the total urine collected while in the intestine there was 77% more *N*B-DGJ than *N*B-DNJ (Fig. 2A). This suggests that *N*B-DGJ passed more slowly out of the gastrointestinal (GI) tract relative to *N*B-DNJ. There appeared to be no difference in distribution of the two compounds in other tissue (Fig.2B). The serum concentration however differed significantly with a lower level of *N*B-DGJ relative to *N*B-DNJ (Fig. 2C), possibly reflecting the slower uptake of *N*B-DGJ from the GI tract. When adjusted for

differential serum levels *N*B-DGJ was distributed to the tissue more efficiently than *N*B-DNJ (Fig. 2D).

Example 4 – Long Term Distribution of NB-DGJ and NB-DNJ in Mouse Serum and Liver

To assay the steady state levels of the compounds when administered long term via the oral route, the concentrations of *N*B-DGJ and *N*B-DNJ in serum and liver were determined by HPLC after treating mice with 2400 mg/kg/day of *N*B-DNJ or *N*B-DGJ (non-radiolabelled) for 5 weeks (see Table 2 below). Both serum and liver concentration of drug were higher in *N*B-DGJ treated mice compared to *N*B-DNJ treated (66 ± 3.1 μM compared to 51 ± 13.3 μM for serum, and 207 ± 30.6 μM compared to 103 ± 21.2 for liver). The level of *N*B-DGJ in liver compared to that of *N*B-DNJ suggests that *N*B-DGJ is selectively taken up into the liver as compared to *N*B-DNJ. Thus, *N*B-DGJ may enter tissues more efficiently and persist longer than *N*B-DNJ.

Table 2 - Concentration of *N*B-DGJ and *N*B-DNJ in serum and liver: Mice were treated with 2400 mg/kg/day of *N*B-DGJ or *N*B-DNJ for 5 weeks (n=2), and the compound concentration in serum and liver was then determined by duplicate runs on HPLC.

	Compound concentration (μM)	
	Serum	Liver
*N*B-DGJ	60 ± 3.1	207 ± 30.6
*N*B-DNJ	51 ± 13.3	103 ± 21.2

Example 5 – Depletion of GSL by NB-DGJ and NB-DNJ

The degree of GSL depletion in liver after 10 days or 5 weeks of treatment was compared between mice administered *N*B-DGJ or *N*B-DNJ. The livers were chloroform: methanol-

extracted, gangliosides were analysed by thin layer chromatography and the G_{M2} band intensity was quantitated by densitometry. The relative G_{M2} concentrations (compared to control mice) in livers of mice treated with a range of *N*B-DGJ or *N*B-DNJ doses (300-4800 mg/kg/day) for 10 days show a dose-dependent response to both compounds (see Fig. 3A). There was no significant difference between the G_{M2} depletion achieved by the two compounds at any of the concentrations tested. After longer treatment (2400 mg/kg/day for 5 weeks), the G_{M2} concentrations in livers of mice treated with *N*B-DNJ or *N*B-DGJ were reduced to 35 ± 4% and 26 ± 11%, respectively, in relation to the concentration in control livers (see Figs. 3B and C).

Thus, both analogues (*N*B-DNJ and *N*B-DGJ) were shown to be potent inhibitors of GSL biosynthesis *in vivo*. After 10 days of treatment, dose-dependent GSL depletion was seen in livers of mice fed either *N*B-DNJ or *N*B-DGJ. The lowest dose causing GSL depletion was 600 mg/kg/day (25% reduction). The highest dose evaluated (4800 mg/kg/day) caused 60-70% depletion. Similar data were obtained with both compounds. Although there is a two fold higher concentration of *N*B-DGJ in liver this was not observed when GSL depletion was measured, where both compounds gave comparable inhibition of G_{M2} biosynthesis. This may reflect differential cellular uptake of the compounds into hepatocytes, endothelial cells and Kuppfer cells as G_{M2} may be primarily the product of one cell type whereas the compound could be sequestered in non-G_{M2} synthesising cells. GSL depletion after longer treatment at a dosage of 2400 mg/kg/day was also determined. After 5 weeks of feeding, the G_{M2} concentration was reduced by 74% by *N*B-DGJ and 65% by *N*B-DNJ. The drug distribution and G_{M2} depletion suggest treatment of GSL storage disorders should be as effective with *N*B-DGJ, since it has been shown that *N*B-DNJ reduces storage in mouse models of these diseases and *N*B-DGJ is slightly superior to *N*B-DNJ in inhibiting GSL biosynthesis *in vivo*.

Example 6 – Effects of NB-DGJ and NB-DNJ on Growth and Lymphoid Organ Size

To examine the overall well being of the mice treated with *N*B-DGJ or *N*B-DNJ (2400 mg/kg/day for 5 weeks) the mice were monitored 2-3 times per week, body weights recorded, and the effects of *N*B-DGJ and *N*B-DNJ on growth rates determined (see Fig. 4). The *N*B-DNJ treated mice grew more slowly than untreated control mice, while *N*B-DGJ treated mice showed no difference in growth rates relative to the untreated controls. After 5 weeks of treatment, the *N*B-DNJ mice weighed 25% less than control and the *N*B-DGJ mice. Thymuses and spleens removed from *N*B-DNJ mice were smaller than those of control or *N*B-DGJ mice (see Fig. 5), while the weights of other organs such as liver and kidney were unaffected. Treatment with *N*B-DNJ reduced the thymus weight by 61 ± 2% and spleen weight by 62 ± 3% compared to organs from control mice. In contrast, *N*B-DGJ had no effect on lymphoid organ weight. The loss of body weight in *N*B-DNJ mice did not account for the large reduction in lymphoid organ size. If expressed as a ratio to body weight, the organ weights were still reduced significantly (thymus to body weight ratio was reduced by 45 ± 5% and spleen to body weight ratio by 48 ± 4% in *N*B-DNJ mice compared to controls). It was observed that *N*B-DNJ treated mice had less fat associated with their organs (kidney, spleen etc.) and lacked subcutaneous fat compared to control or *N*B-DGJ treated mice (data not shown).

The fact that loss of body weight and reduction of lymphoid organ size is caused by *N*B-DNJ but not by *N*B-DGJ suggests that these effects are a function of glucosidase inhibition (or an as yet unidentified activity) by *N*B-DNJ, not GSL biosynthesis inhibition (an activity shared by both compounds). The effect of *N*B-DNJ in the present study on the inhibition of glycogen breakdown could provide a possible explanation for at least part of the weight loss observed in *N*B-DNJ treated mice. It was shown that, after 12h of starvation, when the control and *N*B-DGJ treated mice had depleted most of their glycogen, *N*B-DNJ treated mice still had a significant

amount of glycogen in their livers. Both following starvation and between episodes of feeding, the mouse would normally break down glycogen to provide the brain, muscles and other tissues of the body with glucose. However, if glycogenoloysis was partial inhibited, as in the *N*B-DNJ treated mice, the mouse would have to use other fuel sources, such as fat, to meet its energy demand. The store of adipose tissue would decrease with time resulting in reduced body weight. This hypothesis fits with the observation that the *N*B-DNJ treated mice (both fed and starved) had very little subcutaneous fat compared to normal or *N*B-DGJ treated mice. The inhibition of glycogenolysis by *N*B-DGJ is probably due to inhibition of the glycogen debranching enzyme (4-α-glucanotransferase, EC 2.4.1.25 and α-1,6-glucosidase, EC 3.2.1.33). Although never reported for *N*B-DNJ, inhibition of the α-1,6-glucosidase activity of this enzyme has previously been observed for other DNJ-derivatives (Arai et al 1998, *Circulation* **97**(13): 1290-7; Bollen et al *Eur-J-Biochem* **181**(3): 775-80). If this is also the case for *N*B-DNJ, over prolonged treatment periods this could cause (pathological) glycogen storage. If this does occur however, it is exceeding slow storage as animals on drug for prolonged periods in excess of six months show no overt signs of pathology (data not shown). What may be occurring is that the basal level of glycogen is increased due to partial enzyme inhibition, but that this remains relatively constant over time at the doses of inhibitor used in this study.

*N*B-DNJ treated mice had consistently smaller lymphoid organs. However, *N*B-DGJ did not show this effect, again implying that this is not the result of GSL biosynthesis inhibition in animals treated with *N*B-DNJ.

Example 7 – Inhibition of Disaccharidases In Vitro

*N*B-DGJ, *N*B-DNJ and the parental non-alkylated compound DNJ were assessed for their capacities to inhibit the sucrase and maltase activities of the enzyme sucrase-isomaltase (which has disaccharidase activities for the breakdown of sucrose, maltose

and isomaltose). Inhibition of this enzyme by DNJ has previously been reported (Hanozet *et al.*, (1981). *J. Biol. Chem* **256**:3703-3711). Both substrate and inhibitor concentrations were varied and the K_i calculated (see Table 3). *N*B-DNJ and DNJ were found to be potent inhibitors of both sucrase and maltase (K_i (sucrase) = 0.03 μM and K_i (maltase)=0.07 μM for DNJ, and K_i (sucrase) = 0.26 μM and K_i (maltase) = 0.37 μM for *N*B-DNJ), while *N*B-DGJ was less potent (K_i (sucrase) = 2 mM, (maltase) non-inhibitor at 2 mM).

*N*B-DNJ, DNJ, *N*B-DGJ and DGJ were also tested for their capacity to inhibit lactase (Fig. 6 and Table 4). DNJ, *N*B-DGJ and DGJ all inhibited lactase (K_i of 13 μM, 30 μM and 85 μM for DNJ, DGJ and *N*B-DGJ, respectively). Lactase inhibition by *N*B-DNJ was very weak (K_i = 4 mM).

Table 3 – K_is for the inhibition of sucrase and maltase by DNJ, *N*B-DNJ and *N*B-DGJ. NI (non-inhibitory at 2mM).

	K_i (μM)	
DNJ	*Sucrase*	*Maltase*
	0.03	0.07
NB-DNJ	0.26	0.37
NB-DGJ	2000	NI

Table 4 - K_is for the inhibition of lactase by DNJ, *N*B-DNJ, DGJ and *N*B-DGJ.

DNJ	*K_I (μM)*
	13
NB-DNJ	4000
DGJ	30
NB-DGJ	85

The primary side effect of NB-DNJ has been observed to be osmotic diarrhoea. The diarrhoea is thought to be caused by inhibition of disaccharidases in the intestine, which means that sugars like sucrose and maltose cannot be catabolised and absorbed from the digestive system. Sucrose consists of one glucose and one fructose residue, and maltose of two glucose residues. It is therefore not surprising that the results in this example show that the glucose analogues NB-DNJ and DNJ are very potent inhibitors of the sucrase and maltase activity while the galactose analogue NB-DGJ is not inhibitory. It was found that DNJ, NB-DGJ and DGJ all inhibited lactase, but the K_is were at least 10^2 times higher than for sucrase and maltase inhibition by the glucose analogues. NB-DNJ, however, was not a good inhibitor of lactase (K_i 4mM). In practical terms this means that NB-DGJ might be best tolerated on a lactose-free diet, but should not interfere with the digestion of other carbohydrates. The lack of side effects associated with NB-DGJ in vivo may have important implications for the potential treatment of infants and young children where these side effects could reduce tolerability to a greater extent than those experienced in adults.

Thus it can be seen that *N*B-DGJ has been shown to deplete GSL *in vivo* and to exhibit far fewer *in vitro* and *in vivo* enzyme inhibitory properties than *N*B-DNJ, making this a more selective compound. Of the activities listed below in Table 5, lactase inhibition is the only one associated with *N*B-DGJ and is probably the simplest to overcome by restricting dietary intake of lactose.

Table 5

	NB-DNJ	*NB-DGJ*
GSL Biosynthesis	+	+
Weight loss	+	-
Lymphoid organ reduction	+	-
ER α-glucosidase I and II inhibition*	+	-
Sucrase and maltase inhibition**	+	-
Lactase inhibition***	-	+

* Platt et al (1994) *J Biol Chem* **269**(43): 27108-14

** K_I (sucrase) = 0.26μM, K_I (maltase) = 0.37 μM for NB-DNJ

*** K_I (lactase) = 85 μM for NB-DGJ

samples (shown in parentheses).

CLAIMS:

1. The use of an inhibitor of glycolipid and an agent capable of increasing the rate of glycolipid degradation in the manufacture of a medicament for the treatment of a disorder which has at least a component based on glycolipid storage.

2. A product comprising an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation as a combined preparation for simultaneous, sequential or separate use in the treatment of a disorder which has at least a component based on glycolipid storage.

3. The use as claimed in claim 1 or product as claimed in claim 2, wherein the inhibitor comprises an imino sugar-structured inhibitor of glycolipid, especially glucosylceramide, synthesis.

4. The use or product as claimed in any preceding claim, wherein the inhibitor comprises one or more of N-butyldeoxynojirimycin, N-butyldeoxygalactonojirimycin and N-nonyldeoxynojirimycin.

5. The use or product as claimed in any preceding claim, wherein the inhibitor comprises one or more of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and structurally related analogues thereof.

6. The use or product as claimed in any preceding claim, wherein the inhibitor comprises one or more of a nucleic acid coding for a protein or peptide capable of inhibiting glycolipid synthesis, and an antisense sequence or catalytic RNA capable of interfering with the expression of enzymes responsible for glycolipid synthesis.

7. The use or product as claimed in any preceding claim, wherein the agent capable of increasing the rate of glycolipid degradation comprises one or more of an enzyme which degrades glycolipids, a molecule which increase the activity of such an enzyme, a nucleic acid sequence (DNA or RNA) which codes for such an enzyme, and transplanted bone marrow.

8. A pharmaceutical composition comprising an inhibitor of glycolipid synthesis and an agent capable of increasing the rate of glycolipid degradation.

FIGURE 1

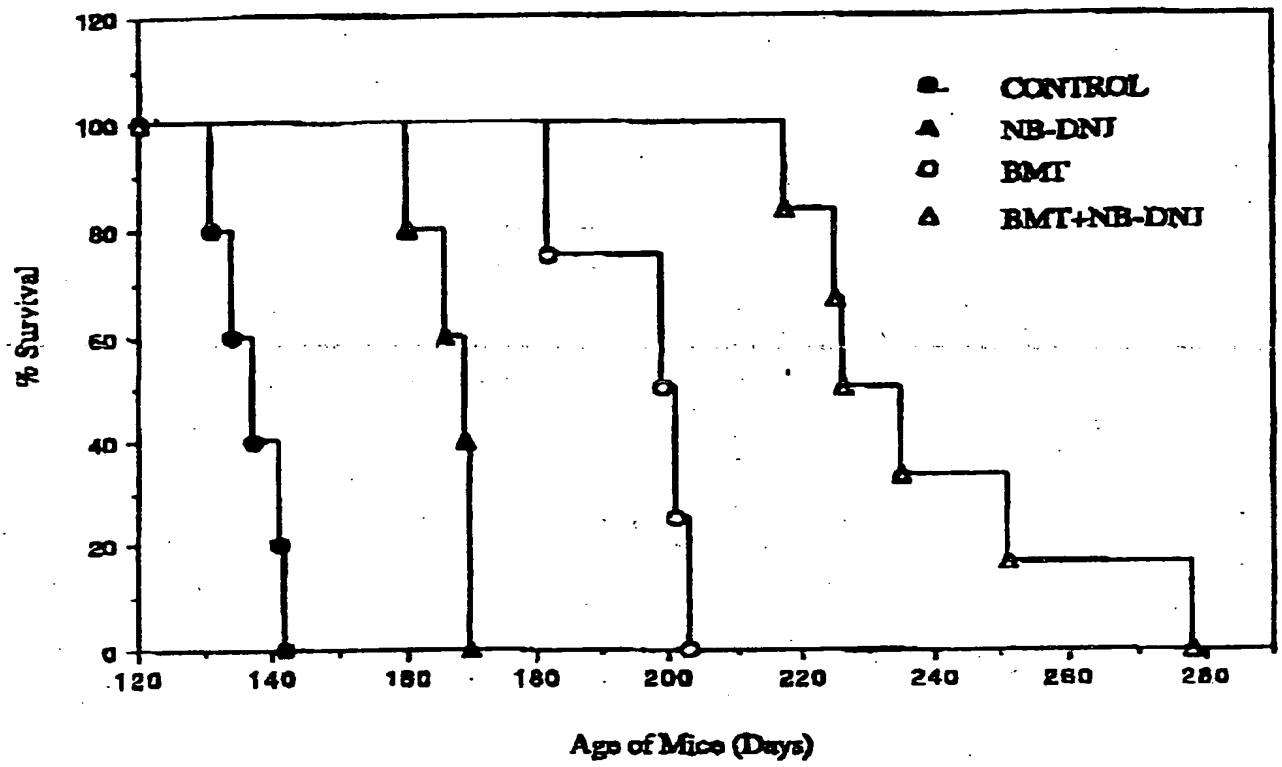

120
100
80
60
40
20
0
% Survival
120
140
160
180
200
220
240
260
280
Age of Mice (Days)
CONTROL
NB-DNJ
BMT
BMT+NB-DNJ

FIGURE 2

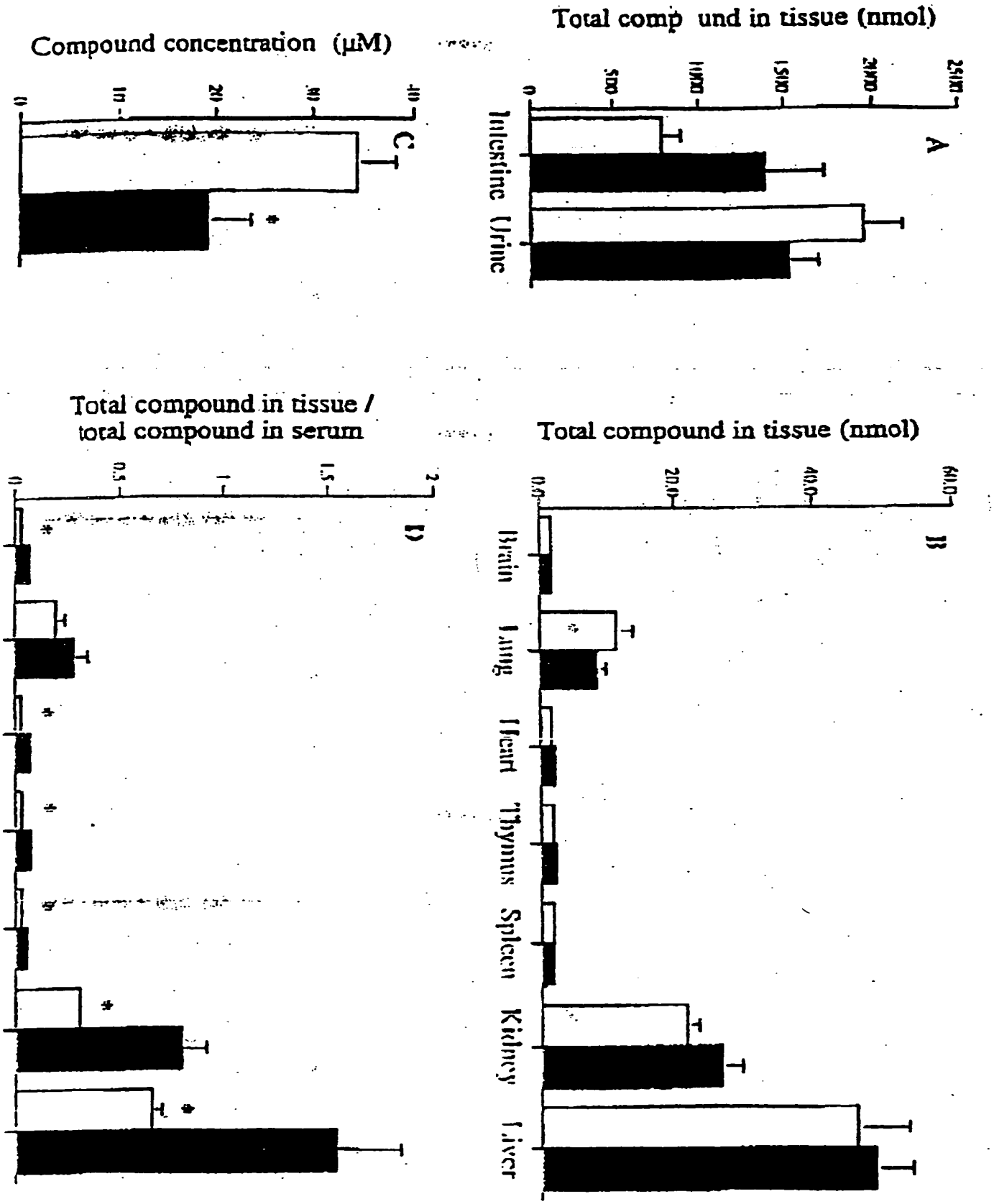
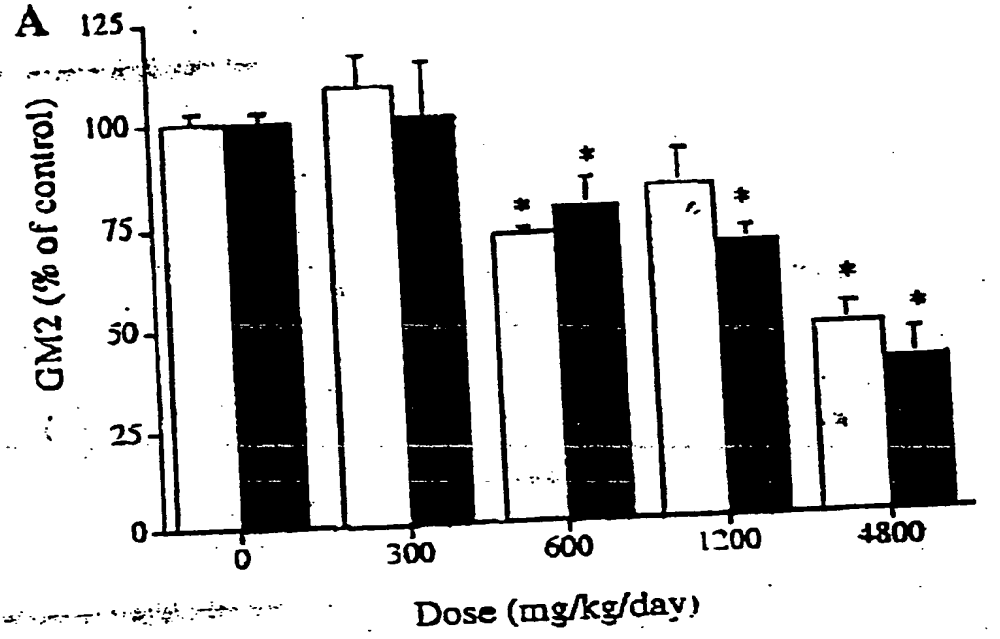
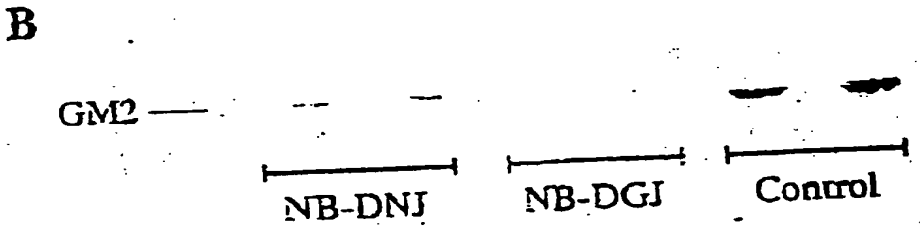

Compound concentration (μM)
C
Total comp und in tissue (nmol)
Intestine Urine
A
Total compound in tissue / total compound in serum
D
Total compound in tissue (nmol)
Brain Lung Heart Thymus Spleen Kidney Liver
B

FIGURE 3


A
125
100
75
50
25
0
GM2 (% of control)
0
300
600
1200
4800
Dose (mg/kg/day)


B
GM2
NB-DNJ
NB-DGJ
Control

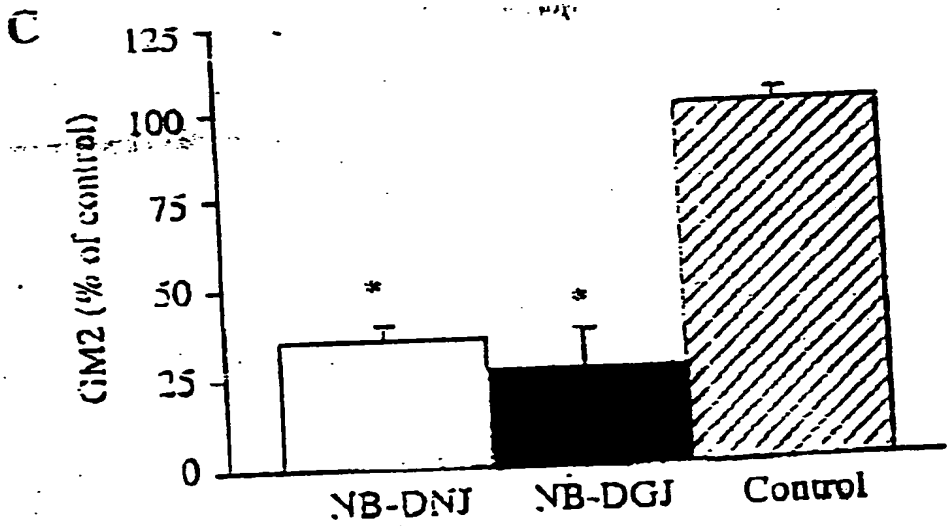

C
125
100
75
50
25
0
GM2 (% of control)
NB-DNJ
NB-DGJ
Control

FIGURE 4

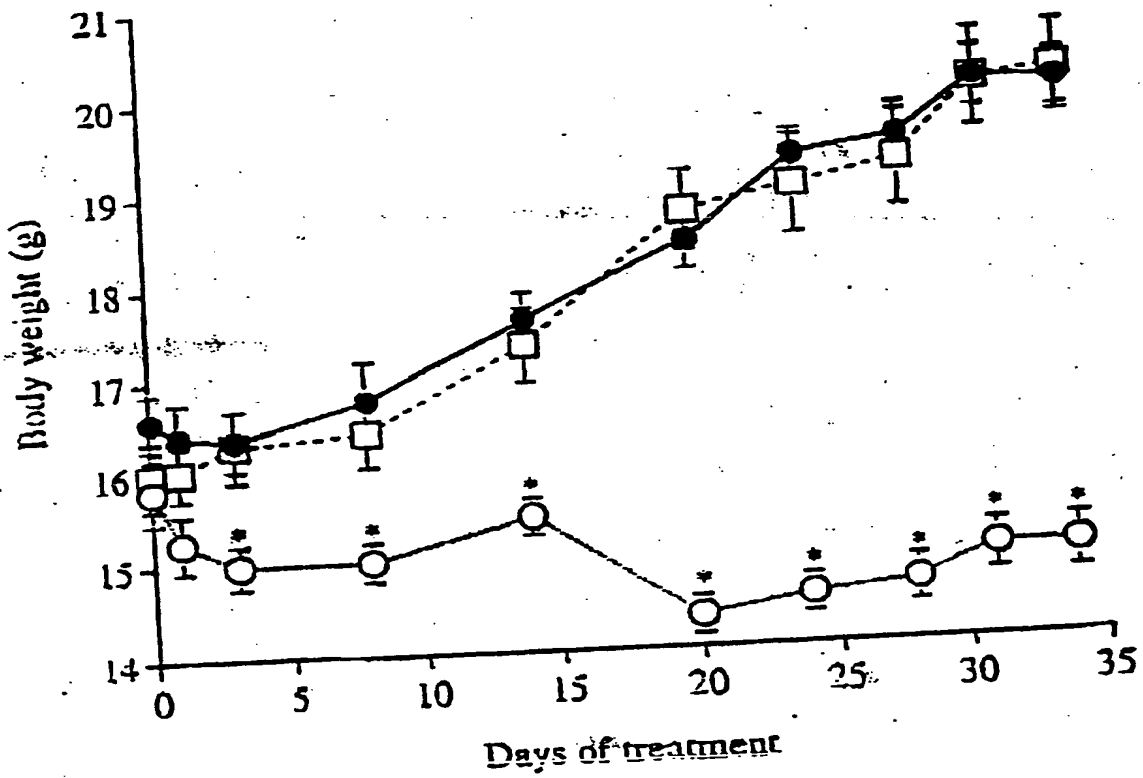

Body weight (g)
21
20
19
18
17
16
15
14
0
5
10
15
20
25
30
35
Days of treatment

FIGURE 5

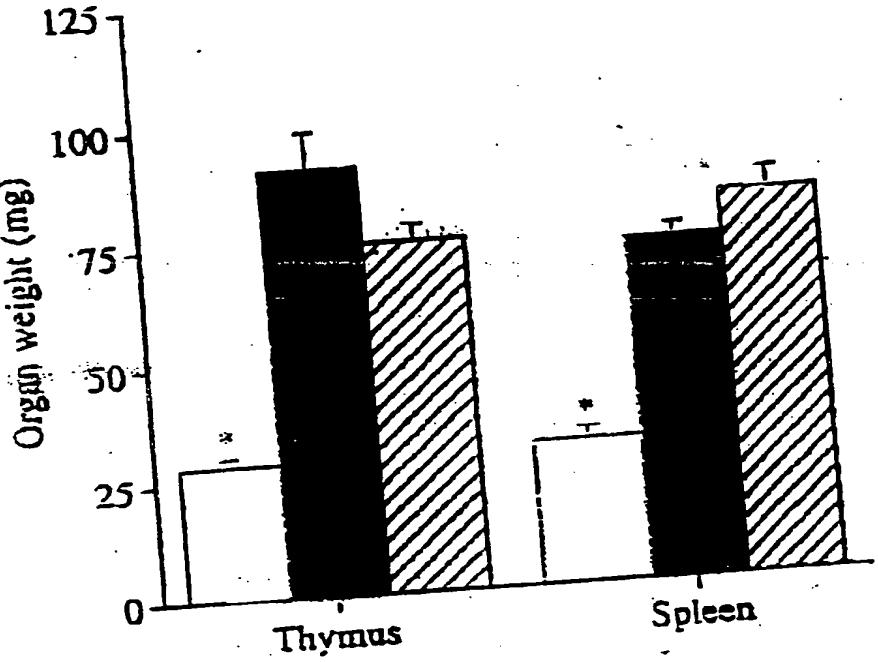
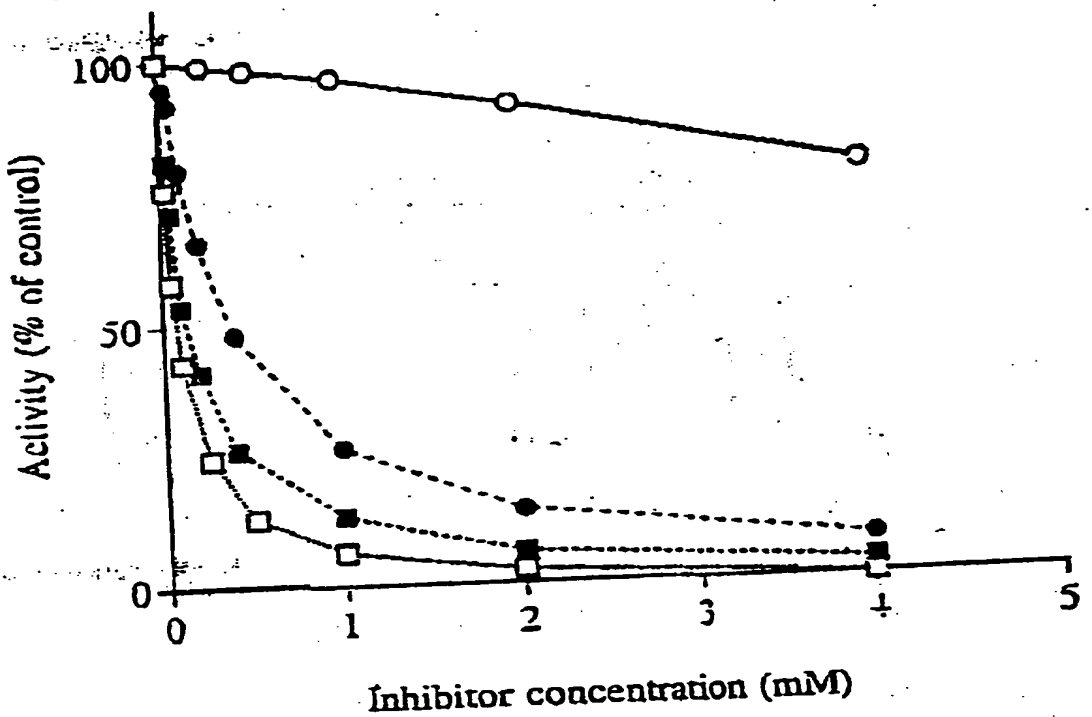


FIGURE 6


100
50
0
Activity (% of control)
0
1
2
3
4
5
Inhibitor concentration (mM)